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Effects of Humic Acid on the Viability and Coagulant Properties of Human Umbilical Vein Endothelial Cells

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We have previously shown that humic acid (well-water humic acid, HA, and synthetic humic acid, SHA) enhances cell surface expression of tissue factor (TF). Here we report that incubation of human umbilical vein endothelial cells (HUVEC) for 2 hr with HA or SHA cause a rapid rise in TF mRNA levels, as shown by Northern blot analysis.

To understand the cytotoxic and fibrinolytic effects of HA and SHA on cultured HUVEC, the cells were treated with varying concentrations of HA and SHA for various periods of time. Both HA and SHA (10–200 μg/ml) inhibited the viability of subconfluent HUVEC, cultured in the presence or absence of 20% FBS (Fetal Bovine serum) in the culture medium, in a dose-dependent manner. Both HA and SHA induced surface changes in the HUVEC as revealed by scanning electron micrography (SEM). However, protocatechuic acid, the monomer of SHA, did not significantly inhibit cell growth, and showed a cytotoxic effect only at 200 μg/ml. Furthermore both HA and SHA stimulated HUVEC to produce plasminogen activator inhibitor (PAI-1) and tissue plasminogen activator (t-PA) in a dose and time dependent fashion; the amount of PAI-1 produced was found to exceed that of t-PA. The monomer of SHA did not have this stimulatory effect. These results distinctly suggest that in addition to the inhibition of viability HA is involved in TF induction and PAI-1 synthesis in HUVEC and these may be some of the plausible mechanisms underlying the thrombotic disorders in Blackfoot disease.

Key words: humic acid, tissue factor, tissue plasminogen activator, plasminogen activator inhibitor, Blackfoot disease

INTRODUCTION

Blackfoot disease is a peripheral vascular disease that is endemic among inhabitants of the southwest coast of Taiwan [1]. Extensive epidemiological studies have disclosed that drinking water from artesian wells contaminated by either humic acid (HA) or arsenic is the most plausible causal factor for Blackfoot disease in the endemic areas [2]. The HA has been shown to shorten the normal human plasma prothrombin time and inhibit plasmin activity [4], induce endothelin production [5], enhance the secretion of plasminogen activator inhibitor [6], enhance the tissue factor activity of cultured endothelial cells [7], inhibit plasma protein C activity [25], and induce blackening of the tail and foot in mice [3]. Unfortunately, no method is available to quantitate the amount of HA in blood and tissue.

We have previously shown that HA and SHA enhance cell surface expression of TF activity. TF is a transmembrane glycoprotein which functions as a cofactor for activation of factor VII and initiation of the extrinsic coagulation pathway. However, expression of TF can be induced in endothelial cells by physical perturbations or in response to stimulation by exogenous growth factors and inflammatory mediators such as fibroblast growth factor, platelet-derived growth factor [8], thrombin [9], interleukin-1 [12], endotoxin [13], tumor necrosis factor [14], and phorbol 12-myristate 13-acetate [15]. Enhanced expression of endothelial cell TF activity may lead to local procoagulant states or thrombotic disorders.

Endothelial cells synthesize and secrete tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI-1) [10]. Second messengers and protein kinases

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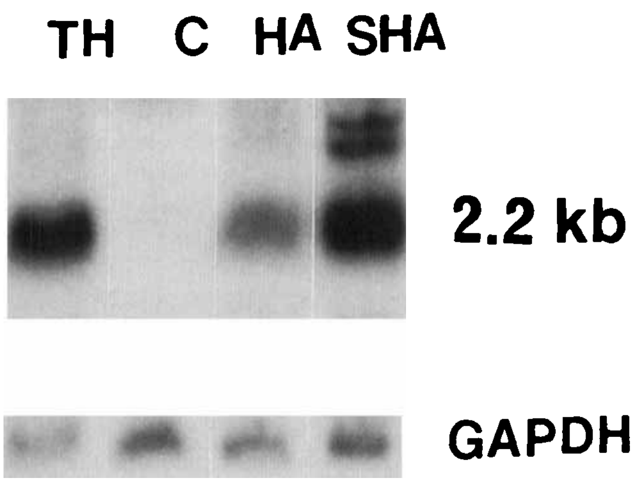

Fig. 1. Effects of HA, SHA, and thrombin on TF mRNA expression in HUVEC. Confluent HUVEC monolayers were incubated in medium containing 20% FBS alone (control cells, C), or medium supplemented with thrombin (TH, 0.5 U/ml), HA (200 μg/ml), or SHA (200 μg/ml) for 2 hr. Total RNA was extracted and 20 μg loaded in each lane. GAPDH mRNA expression was used as an internal control.

are known to be involved in the induction of PAI-1 [28–31] and t-PA [30,31] synthesis. PAI-1 inhibits the generation of plasmin by forming an inactive complex with t-PA, thereby preventing plasminogen activation [11]. The pathological mechanism by which HA promotes intravascular coagulation in vivo is unclear. We have shown in the previous study that HA/SHA can increase the permeability of the HUVEC membrane to extracellular Ca^{2+} ions, leading to the elevation of intracellular Ca^{2+} ion concentration [7]. An increase in the cytosolic Ca^{2+} concentration is often linked to the onset of cytotoxicity, and can directly mediate cell death by causing disruption of the cytoskeleton, DNA fragmentation and extensive damage to other cellular components [26,27]. In this report we characterize the effects of natural well-water HA and synthetic HA (SHA) on the viability, TF mRNA expression and on the production of t-PA and PAI-1 in cultured HUVEC in order to understand the pathogenesis of Blackfoot disease.

MATERIALS AND METHODS

Purification of HA From Well Water

Well water was obtained from Blackfoot disease endemic areas [1]. HA was purified from the drinking well water by absorption chromatography with an XAD-7 (Sigma Chemical, St. Louis, Missouri) column and Sephadex G-25 (Sigma) chromatography according to published methods [17,18].

Preparation and Purification of Synthetic Humic Acid

Humic acid was synthesized following a published procedure, with slight modification [18]. For oxidative polymerization, 1 g of protocatechuic acid in 100 ml of distilled water was oxidized with sodium periodate on a shaker bath for 24 hr at 50°C. The mixture was then centrifuged at 3,000g. The supernatant was acidified to pH 1.0 with 0.1 N HCl and further centrifuged. The precipitate was solubilized in 0.1 N NaOH and purified by absorption chromatography with XAD-7 resin and fractionated by Sephadex G-25 chromatography as described previously [17,18].

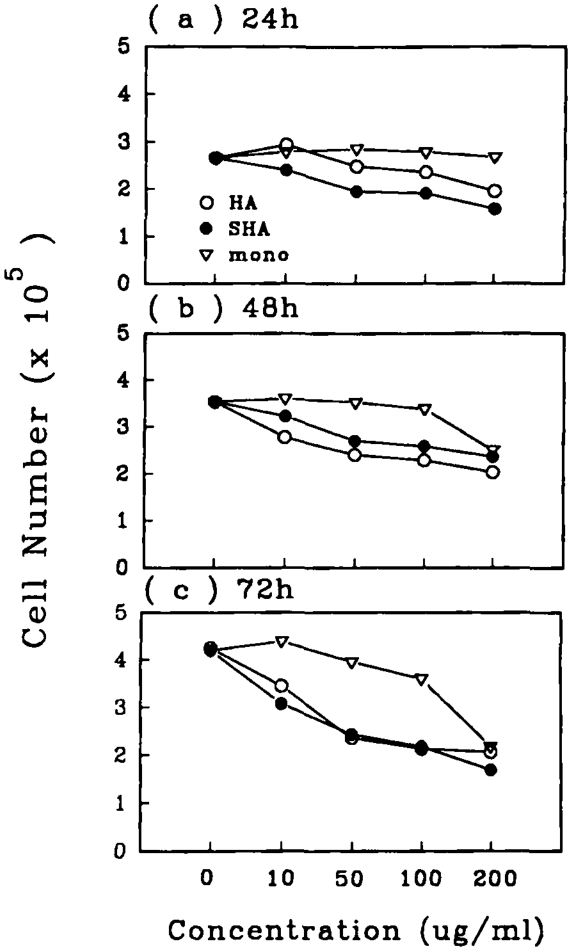

Fig. 2. Effects of various concentrations of HA, SHA, and monomeric protocatechuic acid on the growth of HUVEC. The cells were maintained at 2.3×10^5 cells/well in 6-well plates and cultured in M-199 medium containing 20% FBS for 24 hr before treatment. Cells were harvested after incubation with HA or SHA or the monomer for (a) 24 hr, (b) 48 hr, and (c) 72 hr. Each value is the mean of duplicate cultures.

Isolation, Culture, and Harvest of HUVEC

HUVEC were grown in M-199 containing 20% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml) (GIBCO, Grand Island, NY) [19]. Upon reaching confluence, the cells were detached with trypsin-EDTA and subcultured in 6-well tissue culture plates (Costar) at a density of 2.3×10^5 cells/well in the presence or

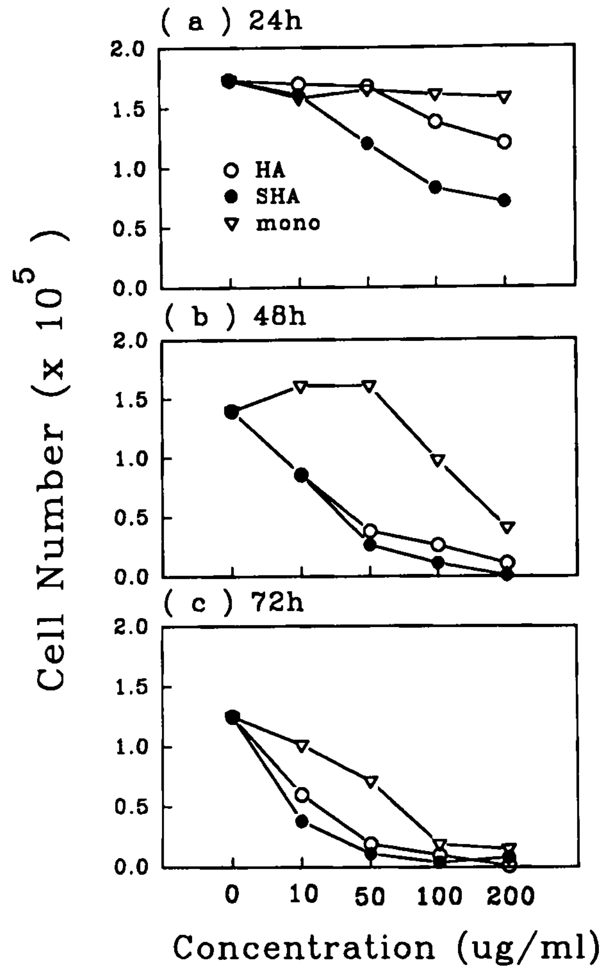

Fig. 3. Effects of various concentrations of HA, SHA, and monomeric protocatechuic acid on the growth of HUVEC. The cells were maintained at 2.3 × 10^5 cells/well in 6-well plates and cultured in serum free M-199 for 24 hr before treatment. Cells were harvested after incubation with HA or SHA or the monomer for (a) 24 hr, (b) 48 hr, and (c) 72 hr. Each value is the mean of duplicate cultures.

absence (serum free) 20% FBS in the culture medium. The serum free medium was without any supplement. Cells were examined for morphological changes by phase-contrast microscope. After assay, the cells were removed from each well with 0.25% trypsin (GIBCO) and counted in a hemocytometer.

TF Messenger RNA (mRNA) Analysis

Total RNA was prepared from 1 × 10^7 cells using 4.0 M guanidinium isothiocyanate containing 35 mM sodium acetate and 0.1% 2-mercaptoethanol, electrophoresed, and transferred onto membranes as previously described [36]. Twenty micrograms of total RNA was loaded per lane and probed with TF cDNA probe, a 830-bp EcoRI fragment of plasmid pGEM1, a kind gift from Dr. S.H. Lin (National Taiwan University, Taipei). The cDNA probes were labeled with [^{32}P]d-CTP by a random primer DNA labelling kit (Boehringer Mannheim, Mannheim, Germany). Hybridization was performed for 16–24 h at 43°C on a water bath. The membranes were washed in 1 × standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) (twice for 15 min), 0.1 × SSC, 0.1% SDS at 65°C. Filters were air-dried and exposed to Kodak XAR film with intensifying screens at −70°C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control.

Preparation of HUVEC for Scanning Electron Micrography (SEM)

Cells were grown on cover slips placed at the bottom of cell culture plates and incubated with 200 μg/ml of HA for 2 hr. The cells were then fixed, dehydrated, desiccated, and sprayed with colloidal gold according to established methods. The changes on the surface of the cells were assessed by counting 500 cells at random under a scanning electron microscope.

Determination of t-PA Antigen and PAI-1 Antigen in HUVEC

The amount of t-PA antigen and PAI-1 antigen in endothelial cell-conditioned medium was determined by a double antibody sandwich enzyme-linked immunosorbent assay using the immunobind TPA kit (American Diagnostics, Inc., Greenwich, Connecticut).

Assay of LDH Activity in HUVEC

Cell toxicity was monitored by measuring the lactate dehydrogenase activity using a Sigma Kit.

Statistics

Data are reported as Mean ± S.E.M., N = 3 to 4 for all groups. The statistical analysis were carried out according to Student's "t" test. A significant level of $P < 0.05$ was used for all comparisons.

RESULTS

Effect of HA/SHA on TF mRNA Content

Exposure of cells for 2 hr to 0.5 U/ml thrombin, 200 μg/ml HA or 200 μg/ml SHA resulted in the induction of a major 2.2 kb TF mRNA transcript, whereas unstimulated cells contained no detectable levels of TF mRNA. Induction of another minor TF transcript of 3.5 and 3.1 kb was occasionally detected (Fig. 1), in agreement with previous observations [33–35].

Effect of HA/SHA on Viability and Growth of HUVEC

HUVEC were cultured with HA, SHA or protocatechuic acid for 24–72 hr at concentrations ranging from 10–200 μg/ml. Both HA and SHA were found to inhibit

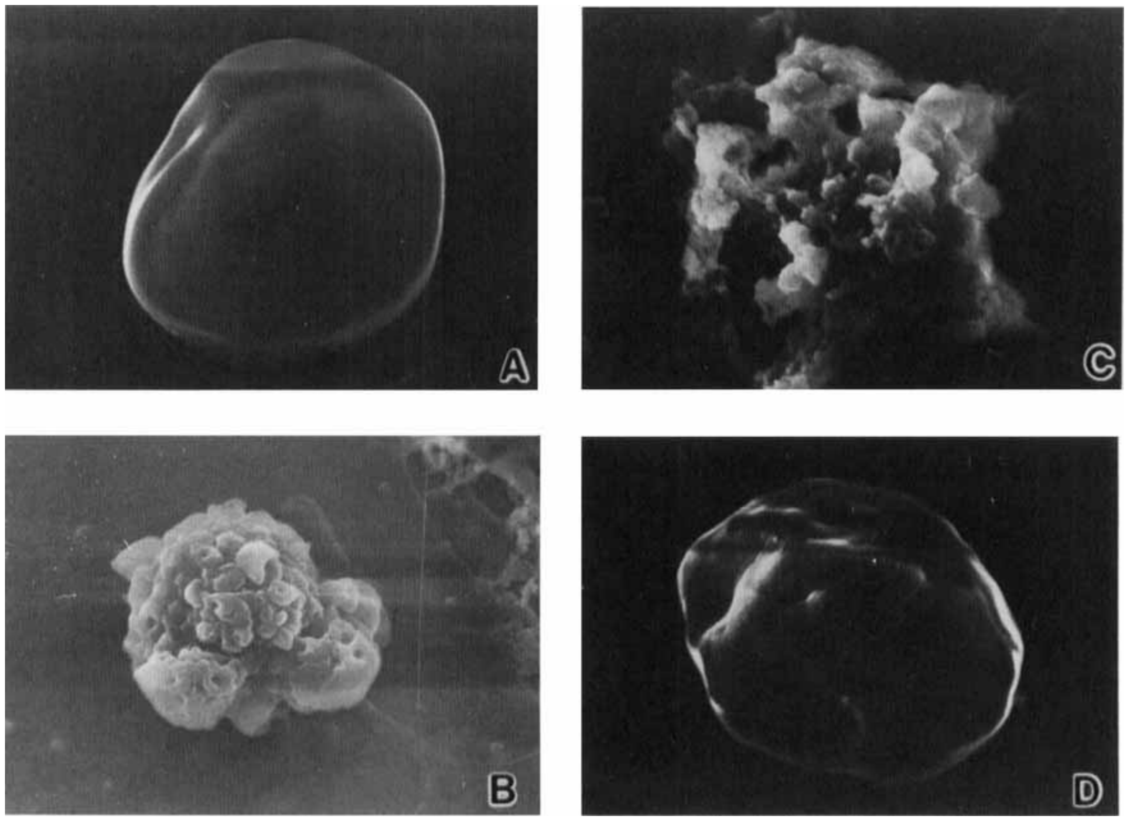

Fig. 4. Scanning electron micrographs of the surfaces of HUVEC. The cells were stimulated for 24 hr with 200 μg/ml of (a) control, ×7,500, (b) HA, ×7,500, (c) SHA, ×10,000, and (d) monomeric protocatechuic acid, ×7,500 in serum free M-199.

the growth of subconfluent HUVEC in 20% FBS M-199 in a dose dependent manner (Fig. 2). A 50% inhibition of cell growth was noted at a concentration of 50 μg/ml of HA or SHA, and there was almost no increase in the cell number at a concentration of 200 μg/ml of HA or SHA. High concentrations (200 μg/ml) of protocatechuic acid was found to be cytotoxic possibly due to autopolymerization of the monomer. Furthermore, the effect of HA or SHA or protocatechuic acid was tested on HUVEC in serum-free M-199 medium. The viability and number of cells were measured after incubation for 1–3 days (Fig. 3). Cell numbers of all 72 hr cultures including those of control declined substantially and no viable cells were observed in the cultures treated with high concentrations of HA and SHA (>50 μg/ml), indicating the cytotoxic effect on HUVEC, as shown in Figure 4, the cell surface of HUVEC was damaged upon incubation with HA and SHA in serum-free M-199 for 24 hr (Fig. 4b,c).

Effect of HA/SHA on Production of t-PA and PAI-1 Antigen by HUVEC

The basal level of t-PA and PAI-1 antigen in the culture medium at 24–72 hr was 1.7–4.8 ng/10^5 cells. When endothelial cells were stimulated with HA or SHA for 3 days, the amount of t-PA antigen was found to decrease. Protocatechuic acid however did not cause any reduction in the amount of t-PA antigen (Fig. 5). HA and SHA significantly increased the PAI-1 antigen production of subconfluent HUVEC by 4.5 fold upon 24–72 hr incubation, but protocatechuic acid did not have this ability (Fig. 6). Our finding that HA or SHA increased PAI-1 antigen production and decreases t-PA antigen production indicates that HA or SHA might play an important role in the regulation of fibrinolysis of HUVEC. The concentrations of HA or SHA used in these experiments did not significantly affect LDH release ($P > 0.05$) from HUVEC (data not shown). The trypan blue exclusion assay showed that more than 95% of cells were viable.

DISCUSSION

Blackfoot disease is a peripheral arterial occlusive disease highly prevalent in the southwest coast of Taiwan. The pathological mechanisms of this disease are still unknown. However, HA has been implicated as one of the causative factors. Therefore we designed this study

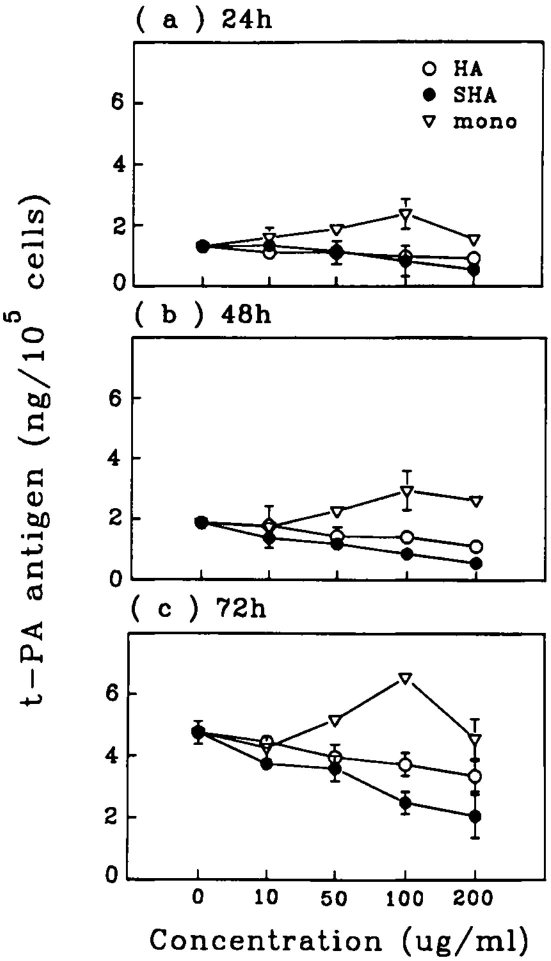

Fig. 5. Effects of various concentrations of HA, SHA, and monomeric protocatechuic acid on t-PA antigen production of HUVEC during incubation for (a) 24 hr, (b) 48 hr, and (c) 72 hr. Cells were maintained at 2.3 × 10^5 cells/well in 6-well plates and cultured in M-199 medium containing 20% FBS for 24 hr before treatment. The data are mean ± S.E.M. of two separate experiments. Each value is the mean of duplicate cultures.

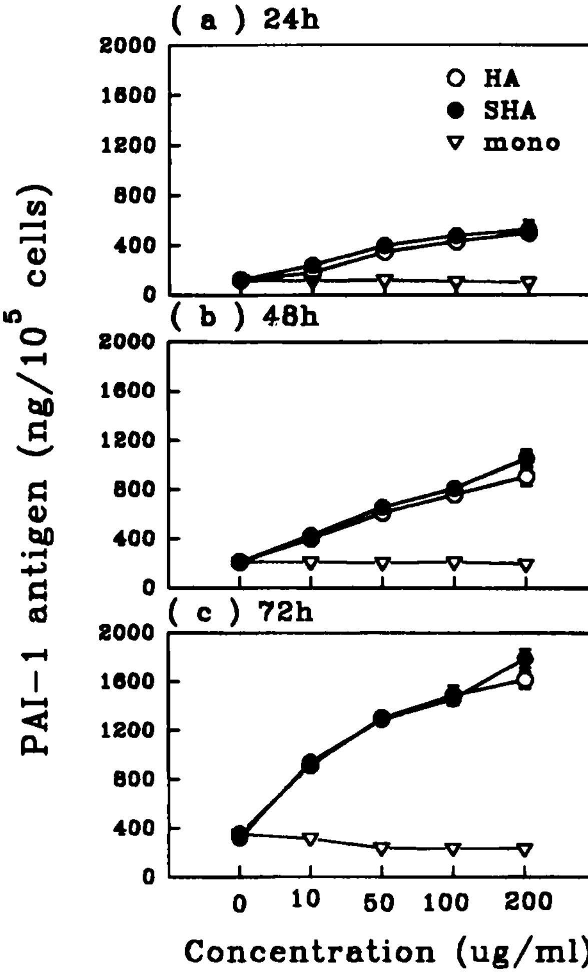

Fig. 6. Effects of various concentrations of HA, SHA, and monomeric protocatechuic acid on PAI-1 antigen production by HUVEC during incubation for (a) 24 hr, (b) 48 hr, and (c) 72 hr. Cells were maintained at 2.3 × 10^5 cells/well in 6-well plates and cultured in M-199 medium containing 20% FBS for 24 hr before treatment. The data are mean ± S.E.M. of two separate experiments. Each value is the mean of duplicate cultures.

to investigate the effects of well-water HA and synthetic HA on the growth and fibrinolytic properties of HUVEC. In a previous study we have demonstrated that HA and SHA enhance cell surface TF expression. PKC has been implicated in the cellular processes leading to tissue factor synthesis [16]. Enhanced TF expression on the surface of endothelial cells may lead to the local procoagulant state or thrombotic disorders. In the present study we demonstrate that HA/SHA induces a rise in TF mRNA levels within 2 hr. The rapid HA/SHA action in induction of TF mRNA levels and TF activity, the transient nature of the induction, and the absence of a requirement for new protein synthesis suggest that the increase in TF mRNA expression in HUVEC represents an early transcriptional event (increased gene transcription and/or increased mRNA stability).

Regulation of intracellular Ca^{2+} concentration is crucial for the regulation and survival of cells. A disruption of Ca^{2+} homeostasis leading to a sustained increase in cytosolic Ca^{2+} level associated with cytotoxicity has been reported in response to a variety of agents in different cell types. Several biochemical mechanisms have been identified that appear to be generally involved in Ca^{2+}-dependent cell injury and death [32]. Both HA and SHA substantially increase the intracellular Ca^{2+} by non-specific interaction with cell membranes [7]. In consistence with these reports both HA and SHA were found to reduce the viability of HUVEC after 24, 48, and 72 hr at 10–200

μg/ml range. SHA however proved to be more cytotoxic to HUVEC. In addition the cell membrane blebbing observed in the electron micrographs of HA/SHA treated cells can also be attributed to the perturbation of the cytoskeleton due to the influx of Ca^{2+}. It is important to note that the toxic effects of HA/SHA were highly pronounced when the cells were treated with the reagents in serum free medium where as the toxicity of HA/SHA was reduced in the presence of 20% FBS, indicating to some unidentified protective effect of serum.

Regulation of intravascular fibrinolysis operates partly at the level of circulating t-PA and PAI-1 [20]. Derangements leading to endothelial cell injury can cause cellular dysfunction resulting in abnormal hemostasis and in thrombotic and hemorrhagic disorders in man [21]. Recent studies have shown that an excessive production of PAI-1 over t-PA, can be induced by thrombin [22], endotoxin [23], and TNF [24]. Second messengers and protein kinases are involved in the induction of PAI-1 [28–31] and t-PA [30,31] synthesis. The present study demonstrates that HA and SHA, but not a monomer of SHA, causes a profibrinolytic response in HUVEC, decreasing t-PA production and increasing PAI-1 secretion after 72 hr incubation. Thus these results suggest that secondary messenger pathways are involved in the activation of intravascular blood coagulation and fibrinolysis, and that vascular endothelial cell damage may partly lead to the thrombotic disorders of Blackfoot disease.

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